Journal of the Lepidopterists' Society
35(4), 1981, 281–289

ARTIFICIAL DIETS AND CONTINUOUS REARING METHODS FOR THE SULFUR BUTTERFLIES *COLIAS EURYTHEME* AND *C. PHILODICE* (PIERIDAE)

O. R. Taylor, Jr., J. W. Grula[1] and J. L. Hayes[2]

Departments of Systematics & Ecology and Entomology,
University of Kansas, Lawrence, Kansas 66045

ABSTRACT. Two artificial diets, one based on alfalfa and the other on lima beans, were used to rear *Colias eurytheme* and *C. philodice* in the laboratory. Preparation of the diets and rearing methods are described. *C. eurytheme* had a higher survivorship on both diets and proved to be the easier species to maintain in long term culture. Despite slightly lower survival of both species on the lima bean diet, it seems to be the more advantageous of the two diets because of ease of preparation and lower cost.

The development of artificial diets and culturing techniques for rearing large numbers of Lepidopterous insects has proliferated over the last fifteen years and produced a large body of literature establishing diets for over 250 species (Singh, 1977). The use of artificial diets has several advantages: primarily it makes possible year-round rearing of large numbers of individuals independently of host plant resources; by following proper procedures, it is possible to raise many individuals in a small space with a minimum risk of loss to disease; additionally, experimentation, transport, and storage are also greatly enhanced with the use of artificial diets. Singh (1977) provides a thorough review of the advantages of rearing insects on artificial diets.

Two artificial diets and culturing methods which can be used for the laboratory rearing of two closely related sulfur butterflies, *Colias eurytheme* Boisduval and *C. philodice* Godart, are reported here. Due to its ubiquity, abundance, and many distinctive characteristics, *Colias* is an intensely studied genus with work ongoing concerning its genetics, behavior, physiology and ecology. Easy laboratory rearing of *Colias* on an artificial diet can greatly facilitate investigations involving this group.

Rearing Procedure

Laboratory cultures of *C. eurytheme* and *C. philodice* were started by placing, individually, young females captured in the wild (which are nearly always mated) in small oviposition chambers. The chamber consisted of two parts: a small round or square pint-size container filled with water and vermiculite in which sprigs of fresh alfalfa (*Med-*

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[1] Present address: Phytogen, 101 Waverly, Pasadena, California 91105.
[2] Present address: Dept. of Biology, Univ. of Iowa, Iowa City 52242.

icago sativa Linnaeus), vetch seedlings (*Vicia villosa* Roth), or plugs of white dutch clover (*Trifolium repens* Linnaeus) were placed; and a clear acetate cylinder about 3.5 inches in diameter and 6–7 inches in height with a one-inch wide strip of screen stapled around both ends. A petri dish lid served as a top for the chamber. The chamber was designed to permit air flow but prevent the female from clinging to the sides.

Maximum oviposition was obtained by placing the chambers containing single *Colias* females under fluorescent or incandescent lights. Egg laying usually started within 24 hours and often continued for 7–10 days. Leaves containing eggs were collected from the host plants every 48 hours and placed in a petri dish containing a moistened piece of paper towel. If eggs were not immediately placed in a food cup, they were stored in a refrigerator at 5–10°C. Eggs could be kept in this way with no detectable loss in viability for one week. A decrease in hatchability and larval viability was frequently noted when eggs were refrigerated for longer periods. Females were set up on new sprigs of the host plant every 48 hours and eggs were collected until the female died, except during the first generation fecundity test with *philodice*, which was based on the first three days of oviposition. Females were fed on 1:4 honey to water solution once each day. Fecundity varied greatly under these conditions, but it was not unusual to obtain 500 eggs from a single female.

Colias are plagued by polyhedral virus and other diseases. In an attempt to control trans-ovariole transmission of virus and other pathogens, eggs were surface decontaminated. Bits of leaves with eggs attached were placed in envelopes made from filter paper and immersed in a dilute solution of sodium hypochlorite (Clorox), consisting of 1 ml Clorox, 99 ml water and one drop of detergent, for 10 minutes and then immediately transferred to 100 ml rinse of tap water for another 10 minutes. The envelopes were then placed on filter paper to facilitate removal of excess water from around the eggs.

Eggs (5–20) were placed in 1.0 oz plastic cups containing about 0.25 oz of diet. Since formaldehyde in the preparation can kill the eggs, they were placed on the paper tab lids of the cups; these were then stored upside down until the larvae completed the first instar. These procedures also minimized exposure of the diet to mold spores and reduced the chance that eggs or young larvae would drown in condensation which occasionally accumulated on the surface of the diet. After the first instar, cups were turned on their sides to allow excess moisture to escape. This retarded the growth of mold.

Larvae were reared on the diet in an environmental chamber which

was maintained at elevated temperatures of 30–35°C and at room temperatures. A 16 hour light : 8 hour dark photoperiod was provided under both conditions. The relative humidity was usually 50–60%.

At room temperature eggs hatched in about 72 hours and larvae pupated in 2½ to 3 weeks. The pupal stage lasted 5 to 7 days, for a total egg to adult interval of 28 days. More information concerning developmental rates is contained in the following section.

Larvae were transferred to new cups of diet in 1½ to 2 weeks. This was necessary because of desiccation of the medium. At this time, no more than five larvae were placed in each cup to ensure food availability and to prevent crowding. Cups were kept on their sides to give the larvae access to diet, to provide a suitable surface on the sides of the cup for pupation and to allow excess moisture to escape.

Once larvae had pupated and the pupae had hardened for at least 24 hours, they were removed from the cups and placed in eclosion chambers. These consisted simply of a piece of plastic window screen rolled and stapled into a cylinder with petri dishes serving as top and bottom. The bottom of each chamber was covered by a thin layer of plaster of paris which was moistened to maintain high humidity. Paper towelling was placed over the plaster to provide a rough surface for adults to grip when eclosing. Usually, pupae were allowed to complete development at room temperature; however, we found that development could be halted with refrigeration at 5–10°C for three weeks with a minimum mortality or loss of vigor.

Newly eclosed adults were given at least an hour for their wings to harden before placing them in a mating cage positioned under a bank of 10, 1500 ma Daylight fluorescent bulbs. We used a cage 4½ × 5′ × 6′, with the top covered with clear mylar plastic and with sides consisting of white sheeting or plastic screen. Two to three hundred individuals could be maintained in this cage at one time. Smaller mating cages have also proven successful (Watt, pers. comm.). Synthetic household sponges saturated with honey water were placed in aluminum pans suspended in the cage daily as a food source for the active adults. The time of day and the number of matings were controlled by turning on the light bank over the cage. The greatest number of matings were obtained when females less than two days old were placed in the cage with 2–4 day old males. Copulations lasted 45 to 75 minutes; so, by monitoring the cage every 30 minutes while the light was on, it was possible to collect all the mating pairs. Females were selected from among the mating pairs (depending on the needs of our experiments) and set up for oviposition. Because of inbreeding depression, we avoided breeding from brother-sister matings or from other crosses among closely related individuals.

If vetch (*Vicia villosa*) or white clover (*Trifolium repens*) is substi-

tuted for alfalfa as the oviposition substrate, a laboratory culture of *Colias* can be maintained year-round with no need for any outdoor resources. Vetch is easily grown indoors and is used readily for oviposition by *C. eurytheme* and *C. philodice*. Vetch has been used by Watt and his students as a year-round host plant; however, extreme vigilance must be maintained to control diseases, and it is sometimes necessary to scrub the entire room with disinfectants if a continuous culture is being maintained (Watt, pers. comm.).

Diet Preparation

The alfalfa diet is a modified version of that developed by C. M. Ignoffo (1963) for the cabbage looper, *Trichoplusia ni* (Hübner) (Noctuidae); and was made up in groups which were combined at the time of preparation. The composition of the groups was as follows:

Alfalfa Diet

	Ingredient	Amount
Group 1	agar	40.0 g
	hot distilled water for dissolving agar	1600.0 ml
Group 2	distilled water	300.0 ml
	cholesterol	0.6 mg
	inositol	0.3 g
	choline chloride	2.0 g
	methyl *p*-hydroxybenzoate	3.0 g
	sorbic acid	3.0 g
	sucrose	35.0 g
	fructose	35.0 g
	wheat germ	70.0 g
	vitamin-free caseine	70.0 g
	dried chopped alfalfa	20.0 g
	biological salt mixture (Wesson modification)	20.0 g
	beta-sitosterol	1.0 g
	safflower oil (55% linoleic acid)	7.5 ml
	linolenic acid	7.5 ml
	40% formaldehyde	4.0 ml
	10% KOH	10.0 ml
Group 3	ascorbic acid	6.0 g
	distilled water to dissolve ascorbic acid	30.0 ml
	vitamin mix	30.0 ml
The vitamin mix was composed of the following:		
	distilled water	200.0 ml
	nicotinic acid	200.0 mg
	calcium pantothenate	200.0 mg
	riboflavin	100.0 mg
	thiamine HCl	50.0 mg
	pyridoxine HCl	50.0 mg
	folic acid	50.0 mg
	biotin	20.0 mg
	vitamin B_{12}	2.0 mg

The diet was prepared in the following manner: The components of Group 2 were added together and thoroughly mixed in a blender. Group 2 was then added to Group 1 after the agar was dissolved in the 1600 ml of hot water. This mixture was thoroughly stirred, and when it had cooled to below 60.0°C, Group 3 was added and again thoroughly stirred. The hot diet was then dispensed quickly into the 1 oz styrene cups, filling each cup approximately ⅓ full with about 8.5 ml (9.0 g) of the mixture. The cups were fitted with lids and stored in a refrigerator. This recipe yielded about 225 cups of diet. The growth of bacteria and fungi on the diet was controlled by the anti-microbial mixture consisting of methyl *p*-hydroxybenzoate, sorbic acid, and formaldehyde, which constituted .35% of the combined diet. The alfalfa served as a feeding stimulant as well as a source of nutrients.

Bean Diet

The second diet is very similar to the modified bean diet established by Burton (1969) for the corn ear worm, *Helothis zea* (Bodie) (Noctuidae). This diet was also made up in separate groups which were subsequently combined. Its constituents were the following:

	Ingredient	Amount
Group 1	agar	35.0 g
	hot distilled water for dissolving agar	700.0 ml
Group 2	wheat germ (not toasted)	100 g
	brewers yeast	64 g
	ascorbic acid	7 g
	sorbic acid	2 g
	methyl *p*-hydroxybenzoate	4 g
	10% formaldehyde	16 ml
Group 3	lima beans (soaked overnight)	200 g
	distilled water	200 ml

The preparation of the bean diet was as follows: Lima beans were soaked in water for 12 to 24 hours, and then they and the rest of the Group 2 ingredients were homogenized and mixed thoroughly in a blender. Group 2 was then added to Group 1, after the agar became completely dissolved, and thoroughly mixed in 700 ml of hot distilled water. This recipe yielded about 250 cups of diet. The diet contains a 1.1% anti-microbial mixture made up of methyl *p*-hydroxybenzoate, sorbic acid, and formaldehyde.

Results

Colias philodice usually takes 1–3 days less to complete development from egg to adult on the diets than does *C. eurytheme.* Devel-

TABLE 1. Survivorship fecundity and mating fitness of *C. eurytheme* and *C. philodice* reared on artifical diets. Ranges are given in parentheses.

	Alfalfa diet*		Bean diet	
	eurytheme	*philodice*†	*eurytheme*	*philodice*
First generation survivorship (per brood)	$\bar{x}$ = 30.8% (8–72%) n = 17	$\bar{x}$ = 25.4% (4–68%) n = 16	$\bar{x}$ = 50.0% (27.5–80%) n = 15	$\bar{x}$ = 37.3% (13.8–48.6%) n = 8
First generation mating fitness (per brood) Males	$\bar{x}$ = 31.7% (0–100%) n = 57	**	$\bar{x}$ = 27.0% (0.0(1)–70.8%) n = 15	$\bar{x}$ = 6.7% (0.0(4)–24.5%) n = 8
Females	$\bar{x}$ = 66.4% (0–100%) n = 50	**	$\bar{x}$ = 45.8% (12.0–50.0%) n = 15	$\bar{x}$ = 8.7% (0.0(2)–27.8%) n = 8
First generation fecundity (per female)	$\bar{x}$ = 292.1 (110–455) n = 9	$\bar{x}$ = 129.3 (14–314) n = 23	$\bar{x}$ = 99.6 (61–140) n = 7	$\bar{x}$ = 74.0 (30–120) n = 9
Second generation survivorship (per brood)	$\bar{x}$ = 66.1% (3–94%) n = 8	$\bar{x}$ = 25.6% (4–54%) n = 12	$\bar{x}$ = 22.8% (4.0–60.0%) n = 6	$\bar{x}$ = 17.4% (0.00(1)–46.7%) n = 7
Fecundity of wild females (per female)	$\bar{x}$ = 268.3 (59–458) n = 19	$\bar{x}$ = 185.4 (15-314) n = 17		

* Survivorhsip for individuals reared on the alfalfa diet was recorded from egg-pupa, while those individuals reared on the bean diet were scored as adults (i.e., survivorship from egg to adult). Survivorship to the adult stage was usually 10–20% lower than that recorded through pupation.

† 1st generation fecundity for *C. philodice* was based on 3 days of oviposition for individuals reared on the alfalfa diet.

** Not recorded.

opmental rates were highly dependent on the temperature at which larvae and pupae were maintained. At room temperature *C. eurytheme* took 18–21 days to pupate, and adult eclosion followed in about 5–7 days. When reared at higher temperatures (30–35°C), pupation occurred in about 14–16 days. Thus, at room temperature the generation length for *C. eurytheme* is approximately 24–28 days and at 30–35°C, it is reduced to 21–22 days. By using refrigeration to "hold" eggs and pupae, we were able to synchronize and delay generations.

Some pertinent parameters indicative of diet suitability for laboratory rearing of *C. eurytheme* and *C. philodice* are summarized in Table 1.

Survivorship on the alfalfa diet varied greatly. Initially, survivorship of *C. eurytheme* was high ($\bar{x}$ = 70%, N = 8 broods); large individuals emerged which mated readily and produced large egg clutches. In subsequent tests on different batches of diet, survivorship was lower, averaging 31% for *C. eurytheme* and 25% for *C. philodice* (Table 1). The latter results were more representative. While second

generation survivorship, fecundity and mating fitness were moderately high for the alfalfa diet, the substantial variance in overall survivorship of individuals on this diet led us to adopt the bean diet for use in our laboratory.

First generation survivorship is quite good on the bean diet, but again there is a difference between *C. eurytheme* and *C. philodice,* with lower survivorship for *C. philodice* (50.5 vs. 37.3%). While second generation survivorship shows a sharp decline for both *C. eurytheme* and *C. philodice,* this decline levels off substantially for subsequent generations (data not shown here).

A comparison of the two diets shows that, while the alfalfa diet is higher in fecundity, mating fitness and second generation survivorship, it is substantially lower in first generation survivorship. The difference in first generation survivorship is actually greater than shown in Table 1, since survivorship on the alfalfa diet was measured to the pupal stage, and usually, only 80–90% of the those reaching this stage become adults. In practice it was found that the greater number of first generation adults obtained with the bean diet was more important in maintaining the cultures than the differences between the diets in fecundity, mating fitness and second generation survivorship.

In *C. eurytheme,* the survivorship and mating viabilities of lab generations stayed high enough to maintain a continuous lab culture for 22 months. *C. philodice* presents greater culturing difficulties since it has lower first generation survivorship and mating viability, which then becomes even lower in the second generation. Because of this, it is necessary to add wild stock to a *C. philodice* culture whenever possible. Adding wild stock is desirable for both species to minimize the inbreeding which is an inevitable consequence of laboratory rearing.

Larvae reared on the diet were generally bluish when compared to larvae reared on natural hosts. The difference is evidently due to a lack of carotenoids in the diet (Rothschild, 1978).

DISCUSSION

In general, the bean diet is preferable to the alfalfa diet for the laboratory rearing of *C. eurytheme* and *C. philodice.* The data indicate that first generation survivorship is greater for individuals raised on the bean diet. However, fecundity, fertility, and mating fitness are greater with the alfalfa diet.

The primary difficulty with the alfalfa diet resides in the quality of the dried alfalfa. The alfalfa we used was obtained directly from commercial alfalfa drying plants. It seemed to vary greatly in its ability to

stimulate feeding, possibly because of the varieties of alfalfa, time of year of harvest, or contamination of fields by weed species which could contain feeding deterrents. It is also possible that some of our alfalfa sources contained natural or synthetic additives which were toxic to developing larvae. Another problem is that the alfalfa diet contains a large number of more or less purified sources of proteins, lipids, carbohydrates and vitamins, which must all retain their individual qualities or the quality of the diet is reduced. Additionally, the alfalfa diet is more expensive because of the large number of costly components it requires. In contrast, the bean diet utilizes mostly natural plant derivatives (beans and wheat germ) and yeast, which can be stored for a longer time without deterioration. Finally, because the bean diet is composed of fewer components, it can be prepared more quickly. We routinely prepared an entire batch of bean diet (about 250 cups) in 1–2 hours; whereas, it usually took 2–3 hours to prepare a similar quantity of the alfalfa diet.

Dehydration of the medium is a problem with both diets. The water content must be high enough to prevent desiccation of early instars and permit easy consumption. Larvae which have been raised on a diet with low moisture content often have difficulty emerging from the pupal case and many crippled adults are the result. Transferring larvae to new cups when the diet has become dehydrated and maintaining high humidity in the rearing chamber does much to eliminate this problem. Spraying pupae lightly with water several times a day often reduces adult eclosion problems.

Generally, *C. eurytheme* utilized both diets more successfully than *C. philodice*. We have no explanation for this, other than to speculate that there is some minor nutritional deficiency which resulted in lower *C. philodice* survivorship and viability. Another problem with *C. philodice* involved its low frequency of mating, which may in part have been due to an alteration in the male pheromones used in courtship. One of us (J.W.G.) has found that *C. philodice* males produce substantially smaller amounts of the three esters unique to this species when reared on the diet. *C. eurytheme* was not similarly affected (Grula et al., 1980).

Neither diet is complete, and since it is likely that survivorship, viability and fecundity are greater on natural hosts, both could be substantially improved. Nevertheless, both the alfalfa and bean diets have proven highly successful for mass rearing *C. eurytheme* and *C. philodice*. The alfalfa diet was used to raise large enough numbers of *C. eurytheme* and *C. philodice* to establish the inheritance of ultraviolet reflectance patterns characteristic of the dorsal wing surface of male *C. eurytheme* and *C. philodice* (Silberglied & Taylor, 1973).

The bean diet has been used to determine the genetic basis of male *C. eurytheme* and *C. philodice* pheromone production (Grula & Taylor, 1980) and inheritance of responses to those pheromones by females (Grula & Taylor, 1980).

Finally, we wish to point out that the bean diet has proven to be successful on a trial basis for rearing several other *Colias* species, including *C. alexandra* Edwards and *C. meadii* Edwards, and also the small pierid *Nathalis iole* Boisduval. We suspect that the bean diet, as formulated in this paper, with slight modifications, probably could be used for a wide variety of legume and composite-feeding butterflies. Its general use by a large number of noctuid moth species has already been demonstrated (Shorey & Hale, 1965).

ACKNOWLEDGMENTS

Development of these diets was facilitated by several grants from the General Research Fund of the University of Kansas. We wish to thank Robert L. Burton of Oklahoma State University for suggesting that we should examine the suitability of the lima bean diet.

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Journal of the Lepidopterists' Society
35(4), 1981, 290–296

DESCRIPTION OF THE MATURE LARVA AND NOTES ON *HOLOCHROA DISSOCIARIA* (HULST) (GEOMETRIDAE: ENNOMINAE)[1]

ROGER L. HEITZMAN[2]

Maryland Center for Systematic Entomology, Department of Entomology, University of Maryland, College Park, Maryland 20742

ABSTRACT. The mature larva of *Holochroa d. dissociaria* is described, with illustrations and photographs included. Notes on the life history are given, and related genera discussed.

Holochroa dissociaria (Hulst) (Figs. 13–14) inhabits the mountainous regions of the southwestern United States. The nominate subspecies occurs in Arizona and Colorado. Subspecies *varia* Rindge is known from New Mexico and western Texas. Three Mexican species also are recognized (Rindge, 1961, 1971).

Holochroa belongs to the Nacophorini, a new world tribe of 21 genera (Rindge, 1971; Ferguson, 1982). Rindge (1974) divided the tribe into a compact nominate group and a diverse nonnominate group. Of the four genera in the nominate group, *Nacophora* is more specialized and *Betulodes* and *Thyrinteina* more primitive than *Holochroa* on the basis of adult characters, but *Holochroa* is considered to be the most distantly related of these genera (Rindge, 1961). In the Nacophorini, only the larvae of *Nacophora, Ceratonyx* and *Aethaloida* have previously been studied.

MATERIALS AND METHODS

Nine mature larvae were examined. These were reared on juniper from single females collected at the following localities in Arizona: Walnut Canyon 6500′, 6⅓ mi, ESE of Flagstaff, Coconino Co., July 16, 1965, R. W. Poole, five specimens on *Juniperus* spp.; Onion Saddle 7600′, Chiricahua Mtns., Cochise Co., July 16, 1967, J. G. Franclemont, four specimens on *Juniperus pachyplaea* Torr.

Descriptions and drawings are based on these specimens. A Wild M-5 microscope and drawing tube attachment were used in making the illustrations. The larval photograph was taken by Dr. J. G. Franclemont, Department of Entomology, Cornell University. Adult photography and larval illustrations were done by the author. Measurements are based on the average of the available specimens.

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[1] Supported in part by Systematic Entomology Laboratory, IIBIII, AR, SEA, U.S. Dept. Agric., Research Agreement No. 58-32U4-9-57. Scientific Article No. A-2762, Contribution No. 5811, of the Maryland Agricultural Experiment Station, Department of Entomology.

[2] Graduate Research Assistant, Maryland Center for Systematic Entomology, Department of Entomology, University of Maryland, College Park 20742.